Specific Oxidative Cleavage of Carotenoids by VP14 of Maize

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The plant growth regulator abscisic acid (ABA) is formed by the oxidative cleavage of an epoxy-carotenoid. The synthesis of other apocarotenoids, such as vitamin A in animals, may occur by a similar mechanism. In ABA biosynthesis, oxidative cleavage is the first committed reaction and is believed to be the key regulatory step. A new ABA-deficient mutant of maize has been identified and the corresponding gene, *Vp14*, has been cloned. The recombinant VP14 protein catalyzes the cleavage of 9-*cis*-epoxy-carotenoids to form C_{25} apo-aldehydes and xanthoxin, a precursor of ABA in higher plants.

Apocarotenoids, which are compounds derived from the oxidative cleavage of carotenoids, are widely distributed in nature and have important metabolic and hormonal functions in diverse organisms. In Mucoraceous fungi, trisporic acid is a mediator of sexual processes (*1*). Retinal serves as a photosensory pigment in animals (*2*), green algae (3), and *Halobacterium* (*4*). Retinoids, which are vitamin A derivatives, are morphogens in animals (*5*) and have important clinical applications.

Apocarotenoids may be formed by random cleavage caused by photooxidation or lipoxygenase co-oxidation. However, regulation of the synthesis of biologically active apocarotenoids requires a more precise mechanism for their synthesis. Enzymatic cleavage of carotenoids at a specific position of the polyene chain has been proposed as a method for the synthesis of several apocarotenoids. The most definitiv illustration of enzymatic cleavage is the production of β-cyclocitral (C_{10}) from β-carotene by the cyanobacterium *Microcystis* (*6*). In other organisms, however, enzymatic cleavage of carotenoids has been difficult to demonstrate in vitro. For this reason, the cleavage reaction in vitamin A biosynthesis remains controversial. Cleavage of β-carotene by a 15,15'-dioxygenase to produce two molecules of retinal has been reported (*7*, *8*). However, there is also evidence for asymmetric cleavage of β-carotene. After asymmetric cleavage, additional carbons are removed from the larger product, by a mechanism similar to β-oxidation, to form one molecule of retinoic acid (*9*).

Abscisic acid (ABA) is a plant growth regulator involved in the induction of seed dormancy and in adaptation to various stresses, such as drought (*10*). Elevated levels of ABA induced by stress are, in part, responsible for stomatal closure, changes in gene expression, and other plant adaptations to stress.

Since the elucidation of the structure of ABA, its biosynthetic derivation from carotenoids has been proposed (*11*). Labeling experiments with $^{18}O_2$ suggest that ABA is synthesized from a large precursor pool that already contains two of th four oxygens found in the molecule (*12*). Oxygen derived from the hydroxyl and epoxid of neoxanthin or violaxanthin could account for the observed ^{18}O labeling patt rn. In addition, when etiolated bean seedlings wer stressed, there was a decrease in the lev l of these xanthophylls and a corresponding increase in ABA and its catabolites (*13*).

Direct evidence for a cleavage enzyme in ABA biosynthesis is lacking because of the apparent low abundance and lability of the enzyme. However, several features of the cleavage reaction have been inferred by analysis of the later steps in ABA biosynthesis (Fig. 1). The initial C_{15} cleavage product xanthoxin is rapidly converted to ABA in vivo and in vitro (*14*). In cell-free extracts, *trans*-xanthoxin is converted to *trans*-ABA, which indicates that there is no cis/trans isomerization after cleavage (*14*). Thus, the xanthophyll precursor must have a 9-*cis* configuration to produce *cis*-xanthoxin and subsequently ABA, which is biologically active only in the cis form.

ABA biosynthetic mutants have been identified in a variety of plant species

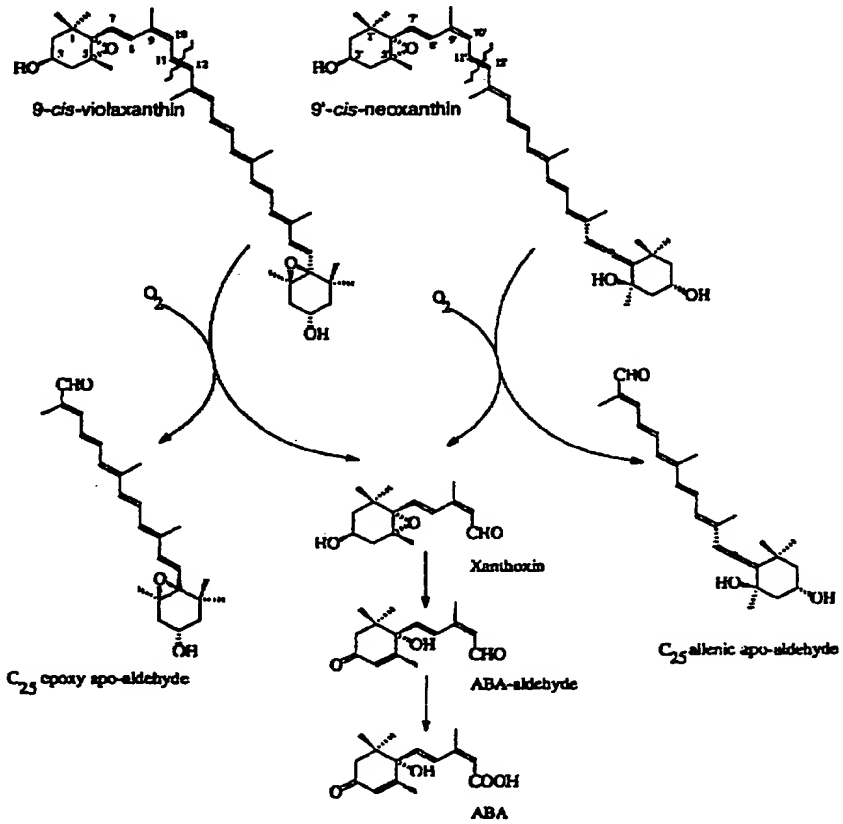

Fig. 1. Proposed pathway of ABA biosynthesis in higher plants.

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(*15*). To date, no mutants impaired in the cleavage reaction have been identified. A new ABA-deficient viviparous mutant in maize, *vp14*, has been isolated and the corresponding gene has been cloned (*16*). This mutant is not impaired in carotenoid biosynthesis or in the conversion of xanthoxin to ABA. The derived amino acid sequence of VP14 shows significant sequence similarity to lignostilbene dioxygenases (LSDs) from *Pseudomonas paucimobilis* (*17*). These LSDs catalyze a reaction similar to the proposed cleavage reaction in ABA biosynthesis. Specifically, a double bond is oxidatively cleaved, yielding two products with aldehyde groups at the site of cleavage.

Using 9-*cis*-violaxanthin as a substrate, the recombinant VP14 protein was tested for cleavage activity (*18*) and the products were analyzed by high-performance liquid chromatography (HPLC) (Fig. 2). The expected cleavage products, xanthoxin and the C_{25} epoxy apo-aldehyde, were identified by their ultraviolet/visible absorption spectra and mass spectra (*19*).

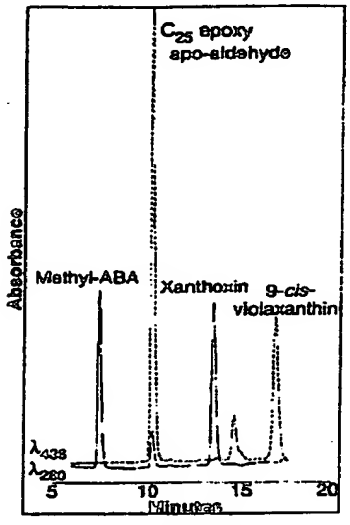

Fig. 2. HPLC chromatogram (*31*) of the cleavage reaction products with the use of 9-*cis*-violaxanthin as a substrate (*32*). Absorbance was measured at 280 nm and 436 nm with a photodiode array detector.

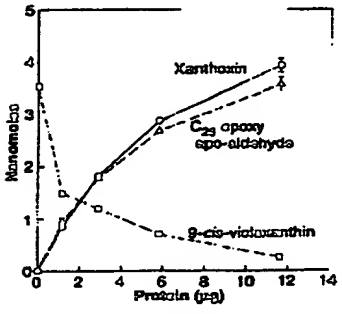

Fig. 3. Decrease in 9-*cis*-violaxanthin and the concomitant increase in xanthoxin and the C_{25} epoxy apo-aldehyde as a function of the VP14 protein concentration. Assays were incubated for 10 min at 22° to 24°C, extracted, and quantified (*31*).

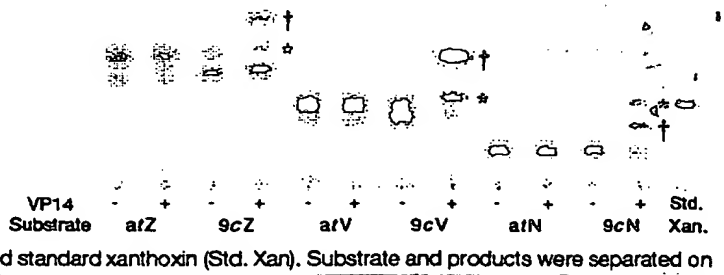

Fig. 4. Thin-layer chromatography of assays with (+) and without (−) VP14 protein. Assays contained approximately 5 μg of the indicated substrat (*32*): the all-*trans* isomers (*at*) and the 9-*cis* isomers (*9c*) of zeaxanthin (Z), violaxanthin (V), neoxanthin (N), and standard xanthoxin (Std. Xan). Substrate and products were separated on a silica gel 60 plate (EM Separations) developed with 10% *iso*-propanol in hexane. The plates were sprayed with 2,4-dinitro-phenylhydrazine to detect xanthoxin and other aldehydes. The C_{25} products are indicated by a dagger and the C_{15} products are indicated by an asterisk. The unlabeled spots are th carotenoid precursors.

Molecular oxygen, ferrous iron, and a detergent were necessary for the cleavage activity (Table 1). A number of organic cofactors were initially added to the assays but none had any effect on the activity (*20*). The cleavage reaction was totally inhibited by EDTA, a chelator of divalent cations (*21*). The removal of EDTA and addition of ferrous iron were sufficient to restore activity. Ascorbate was added to the assays to maintain iron in the proper redox state (*21*).

With increasing VP14 concentration, there was a decrease in 9-*cis*-violaxanthin and an equimolar increase in xanthoxin and the C_{25} epoxy apo-aldehyde (Fig. 3). Whereas photo- or chemical oxidation would result in random cleavage, this stoichiometric conversion of 9-*cis*-violaxanthin to the two products illustrates the specificity of cleavage between the 11 and 12 positions of the polyene chain.

To determine the substrate specificity of the cleavage reaction, the all-*trans* and the 9-*cis* isomers of neoxanthin and violaxanthin were tested. The reaction products were separated on thin-layer chromatography plates and sprayed with 2,4-dinitrophenyl hydrazine to detect aldehydes (Fig. 4). Xanthoxin and the predicted C_{25} products were present only in reactions containing the 9-*cis* isomers. The 9-*cis* isomer of zeaxanthin, formed by iodine isomerization of the all-*trans* zeaxanthin (*22*), was cleaved at the 11-12 position by the VP14 protein (*23*) (Fig. 4). Therefore, it appears that the 9-*cis* configuration was the primary determinant of cleavage specificity for the in vitro assays. Cleavage of 9-*cis*-epoxy-carotenoids results in the production of *cis*-xanthoxin, which will subsequently be converted to the biologically active isomer of ABA in vivo.

Table 1. The requirements for cleavage activity in vitro. This is the standard reaction (*18*) minus the indicated cofactors and 6 μg of VP14 protein.

Triton X-100	Catalase	$FeSO_4$	O_2	Xanthoxin (ng)*
+	+	+	+	865 ± 10
−	+	+	+	n.d.
+	−	+	+	759 ± 32
+	+	−	+	243 ± 14
+	+	+	−	n.d.

*Nanograms of xanthoxin ± SE; n = 2; n.d., not detectable.

The LSDs from *Pseudomonas* (*17*) and VP14 compose a novel class of dioxygenases that catalyze similar double-bond cleavage reactions. The conserved sequences have also been identified in several plant expressed sequence tags, two open reading frames in the genomic sequence of *Synechocystis* (*24*), and a protein expressed in the retinal pigment epithelium of mammals, RPE65 (*25*). The function of these gene products has not yet been determined, but their existence indicates the presence of this class of enzymes in a diverse range of species.

The environment of the carotenoids in the thylakoid and envelope membranes (*26*) is very different from in vitro assays in which the carotenoid substrates are solubilized by detergent. However, the characteristics of the cleavage reaction in substrate specificity and position of cleavage are consistent with the proposed pathway (*27*). Current evidence suggests that this cleavage reaction is the key regulatory step in ABA biosynthesis (*27*). Further characterization of the cleavage reaction and its regulation may allow the manipulation of ABA levels in plants, which would affect such processes as seed dormancy, drought tolerance, and cold hardening.

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18. VP14 was expressed as a glutathione-S-transferase fusion protein (Pharmacia). Enzyme assays contained 100 mM bis-tris (pH 6.7), 0.05% Triton X-100, 10 mM ascorbate, 5 μM $FeSO_4$, catalase (1 mg/ml), and VP14 protein. Assays were incubated for 15 min at 22° to 24°C in a total volume of 100 μl. The appropriate substrate was added in 3 μl of ethanol. The enzyme assay and all subsequent procedures were performed under red light to minimize photooxidation of the precursors and products.
19. The cleavage products were analyzed on a JEOL AX 505 double-focusing mass spectrometer equipped with a Hewlett-Packard gas 5890 chromatograph, using electron ionization at 70 eV. The trimethylsilyl ether derivative of xanthoxin gave a spectrum that was similar to a previously reported spectrum (*28*). The C_{25} apo-aldehydes were introduced via a direct insertion probe heated from ambient temperature to 200°C at 64°C min^{-1}. The fragmentation patterns for the epoxy (5,6-epoxy-3-hydroxy-12′-apo-β-caroten-12′-al) and allenic-(3,5-dihydroxy-6,7-didehydro-12′-apo-β-caroten-12′-al) C_{25} cleavage products were nearly identical to published spectra for these compounds (*29*). Exact mass measurements were performed as described above, with the exception that the instrument's mass resolution was increased from 1000 to 7500. Perfluorokerosene was introduced simultaneously with the sample to provide reference ions for exact mass assignments. The theoretical mass of the isomeric C_{25} cleavage products from neoxanthin and violaxanthin ($C_{25}H_{34}O_5$) is 382.2508. The measured mass of the C_{25} product from neoxanthin was 382.2498, with an error of −2.6 parts per million (ppm) from the calculated mass. The measured mass of the C_{25} product from violaxanthin was 382.2501, with an error of −1.8 ppm.
20. S. H. Schwartz, B. C. Tan, D. A. Gage, J. A. D. Zeevaart, D. R. McCarty, data not shown.
21. Iron was chelated from the VP14 protein with 50 mM EDTA. The EDTA was subsequently removed on a G-25 Sephadex spin column equilibrated with 100 mM bis-tris and 0.05% Triton-X 100. VP14 protein (7 μg) was then added to reactions containing the indicated cofactors [$n = 2$; n.d., not detectable [treatment, xanthoxin (ng) ± SE]]: No iron, n.d.; 5 μM Fe^{2+}, 416.1 ± 8.1; 5 μM Fe^{3+}, n.d.; 5 μM Fe^{3+} + 10 mM ascorbate, 432 ± 8.4.
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23. The low- and high-resolution mass spectra of the C_{25} product from cleavage of 9-*cis*-zeaxanthin were obtained as described above (*19*). For C_{25} zeaxanthin apo-aldehyde (3-hydroxy-12′-apo-β-caroten-12′-al), the mass-to-charge ratio (*m/z*) and relative intensity (in parentheses) were as follows: 366 $[M]^+$ (100), 348 (6), 255 (4), 213 (8), 197 (8), 147 (17), 119 (20), 105 (15), 91 (15). The theoretical mass of the cleavage product ($C_{25}H_{34}O_2$) is 366.2559; and the experimentally determined mass was 366.2564, with an error of 1.5 ppm from the calculated mass. The C_{15} cleavage product was analyzed as described above, except that the probe was heated at 2°C min^{-1}. For C_{15} zeaxanthin apo-aldehyde (3-hydroxy-apo-β-caroten-11-al), *m/z* and relative intensity were as follows: 234 $[M]^+$ (57), 219 (17), 201 (48), 187 (35), 159 (34), 149 (79), 131 (43), 121 (52), 105 (52), 95 (100). The compound ($C_{15}H_{22}O_2$) has a theoretical mass of 234.1620; and the experimentally determined mass was 234.1611, with an error of −3.7 ppm.
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31. After the incubation, 1 ml of H_2O was added to the reactions. The reaction products were then partitioned three times into an equal volume of ethyl acetate with vigorous stirring on a vortex mixer. The ethyl acetate fractions were pooled and dried, and methyl ABA was added as an injection standard. The samples were stored under Ar at −80°C until analysis. Samples were injected on a μPorasil column (Waters) (30 by 0.4 cm) equilibrated with 90% hexane and 10% ethyl acetate. The column was eluted with a linear gradient to 20% hexane and 80% ethyl acetate over 17 min. A standard curve was constructed by injecting known quantities of the compounds analyzed and integrating the peak areas. The integrated peak area of methyl ABA was used to correct for variations in injections. The correlation coefficients were generally greater than 0.99.
32. The carotenoid substrates were isolated from spinach leaves (*30*). The *aba1-5* mutant of *Arabidopsis thaliana* was used as a source of all-*trans*-zeaxanthin, which was isomerized with iodine (*22*) and rechromatographed to isolate 9-*cis*-zeaxanthin.

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Identification of a Chemokine Receptor Encoded by Human Cytomegalovirus as a Cofactor for HIV-1 Entry

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The human cytomegalovirus encodes a β-chemokine receptor (US28) that is distantly related to the human chemokine receptors CCR5 and CXCR4, which also serv as cofactors for the entry into cells of human immunodeficiency virus–type 1 (HIV-1). Like CCR5, US28 allowed infection of CD4-positive human cell lines by primary isolates of HIV-1 and HIV-2, as well as fusion of these cell lines with cells expressing the viral envelope proteins. In addition, US28 mediated infection by cell line–adapted HIV-1 for which CXCR4 was an entry cofactor.

Human immunodeficiency virus infects cells by a process of membrane fusion that is mediated by its envelope glycoproteins (gp120-gp41, or Env) and is generally triggered by the interaction of gp120 with two cellular components: CD4 and a coreceptor belonging to the chemokine receptor family (*1*). The coreceptor for HIV-1 strains adapted to replication in $CD4^+$ cell lines (TCLA strains) was identified by a genetic complementation approach and named fusin (*2*); however, it was later shown to be an α- (or CXC) chemokine receptor and designated CXCR4 (*3*). The isolation of fusin and the antiviral activity of certain β- (or CC) chemokines (*4*) led to the demonstration that the β-chemokine receptor CCR5 is the principal coreceptor for primary HIV-1 strains (*5–8*). In addition to CCR5, certain primary HIV-1 strains (dual tropic) use CXCR4 (*9*), or CXCR4 and CCR2b (*8*), as a coreceptor, whereas others (macrophage tropic) can use CCR3 (*7, 10*). The essential role of CCR5 is nevertheless indicated by the resistance to HIV-1 infection of individuals with defective *CCR5* alleles (*11*). The CCR5 and CXCR4 coreceptors are also used by HIV-2 and the related simian immunodeficiency viruses (*12, 13*).

Several homologs of chemokine receptors are encoded by herpesviruses (*14*); in particular, by the US27, US28, and UL33 open reading frames (ORFs) of the human cytomegalovirus (CMV) (*15*). In fibroblasts infected experimentally, these ORFs were transcribed at a high rate after viral DNA replication (*16*), but their pattern of expression in vivo and their role in the life cycle of CMV are unknown. The product

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